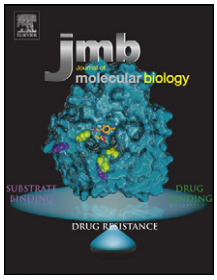

The *Bacillus subtilis* and *Bacillus halodurans* Aspartyl-tRNA Synthetases Retain Recognition of tRNA^{Asn}

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Abstract

Synthesis of asparaginyl-tRNA (Asn-tRNA^{Asn}) in bacteria can be formed either by directly ligating Asn to tRNA^{Asn} using an asparaginyl-tRNA synthetase (AsnRS) or by synthesizing Asn on the tRNA. In the latter two-step indirect pathway, a non-discriminating aspartyl-tRNA synthetase (ND-AspRS) attaches Asp to tRNA^{Asn} and the amidotransferase GatCAB transamidates the Asp to Asn on the tRNA. GatCAB can be similarly used for Gln-tRNA^{Gln} formation. Most bacteria are predicted to use only one route for Asn-tRNA^{Asn} formation. Given that *Bacillus halodurans* and *Bacillus subtilis* encode AsnRS for Asn-tRNA^{Asn} formation and Asn synthetases to synthesize Asn and GatCAB for Gln-tRNA^{Gln} synthesis, their AspRS enzymes were thought to be specific for tRNA^{Asp}. However, we demonstrate that the AspRSs are non-discriminating and can be used with GatCAB to synthesize Asn. The results explain why *B. subtilis* with its Asn synthetase genes knocked out is still an Asn prototroph. Our phylogenetic analysis suggests that this may be common among Firmicutes and 30% of all bacteria. In addition, the phylogeny revealed that discrimination toward tRNA^{Asp} by AspRS has evolved independently multiple times. The retention of the indirect pathway in *B. subtilis* and *B. halodurans* likely reflects the ancient link between Asn biosynthesis and its use in translation that enabled Asn to be added to the genetic code.

Introduction

The fidelity of protein synthesis requires the accurate ligation of amino acids to their cognate tRNAs. The esterification of amino acids to tRNAs is catalyzed by aminoacyl-tRNA synthetases (aaRSs) [1]. Each aaRS is specific for one proteinogenic amino acid and set of tRNA isoacceptors. In many bacterial and archaeal genomes, the asparaginyl-tRNA synthetase (AsnRS) for ligating Asn to tRNA^{Asn} is not encoded [2]. Instead, these prokaryotes synthesize Asn on tRNA^{Asn} using a two-step pathway. First, Asp is ligated to tRNA^{Asn} by a non-discriminating aspartyl-tRNA synthetase (ND-AspRS), an aspartyl-tRNA synthetase (AspRS) with relaxed tRNA specificity [3,4]. The Asp on tRNA^{Asn} is then transamidated to Asn by the amidotransferase GatCAB to form the Asn-tRNA^{Asn} used by the ribosome [5,6]. GatCAB can also be used in bacteria to form Gln-tRNA^{Gln} in species lacking a glutaminyl-tRNA synthetase [2]. First in these organisms, a non-discriminating glutamyl-tRNA synthetase (ND-GluRS) glutamylates tRNA^{Gln} [7] and then GatCAB transamidates the Glu-tRNA^{Gln} to Gln-tRNA^{Gln} [8].

The function of GatCAB in a cell is dictated by which non-discriminating aaRS is expressed (ND-AspRS and/or ND-GluRS). In bacteria such as *Lactobacillus delbrueckii bulgaricus* encoding a discriminating AspRS (D-AspRS) and an ND-GluRS, GatCAB is only for Gln-tRNA^{Gln} formation [9]. In species such as *Deinococcus radiodurans* with a D-GluRS and an ND-AspRS, GatCAB is solely for Asn-tRNA^{Asn} synthesis [6]. In bacteria such as *Staphylococcus aureus* encoding both an ND-GluRS and an ND-AspRS, GatCAB is dual functional [10–12].

Bacterial GatCAB recognizes a U1-A72 base pair in its aminoacyl-tRNA substrates [13,14]. The

presence of a tRNA^{Asn} with U1-A72 while required for the indirect pathway in bacteria for Asn-tRNA^{Asn} formation is not necessarily an indicator that the organism carries out tRNA-dependent Asn biosynthesis. For example, *Escherichia coli* tRNA^{Asn} isoacceptors have a U1-A72 base pair despite *E. coli* lacking GatCAB (Table 1). In addition, *L. delbrueckii bulgaricus*, despite encoding GatCAB along with tRNA^{Asn} isoacceptors with a U1-A72 base pair (Table 1), uses the amidotransferase solely for Gln-tRNA^{Gln} formation as its AspRS is specific for tRNA^{Asp} [9].

The sequence determinants that distinguish D-AspRS enzymes from non-discriminating ones remain unclear [10]. Typically, a bacterial genome is predicted to encode an ND-AspRS when GatCAB is coded for but AsnRS is not. However, it has been established that certain bacteria encode both routes for Asn-tRNA^{Asn} formation. *D. radiodurans*, *Thermus thermophilus*, and *Clostridium acetobutylicum* accomplish this by encoding more than one AspRS enzyme in addition to GatCAB and AsnRS (Table 1) [3,6,15–19]. The additional AspRS enzymes were acquired via horizontal gene transfer from archaea and are non-discriminating, while the lone bacterial-type AspRS in each bacteria is discriminating. The archaeal ND-AspRS with GatCAB in these bacteria is used for tRNA-dependent Asn biosynthesis. Synthesis of Asn on tRNA^{Asn} is the sole means these bacteria have to make the amino acid as these three species lack a functional Asn synthetase (AsnA or AsnB) for tRNA-independent Asn biosynthesis [3,6,15–19].

Recently, we demonstrated that *S. aureus*, *Bdellovibrio bacteriovorus*, and *Legionella pneumophila* also encode both direct and indirect routes for Asn-tRNA^{Asn} formation [10,20]. The two-step indirect pathway provides the sole means Asn synthesis in these species. However, in contrast to *D. radiodurans*, T. *thermophilus*, and *C. acetobutylicum*, these three bacteria each encode only one AspRS. In each bacterium, the lone AspRS serves as the non-discriminating enzyme for tRNA-dependent Asn biosynthesis. The AsnRS in these species is thought to take advantage of the Asn recycled from protein degradation and/or available from the environment. The indirect two-step pathway provides a mechanism to synthesize a basal level of Asn for translation and directly couple Asn biosynthesis with its use in translation. Accordingly, encoding two distinct routes for Asn-tRNA^{Asn} synthesis may enable these bacteria to exploit different environments [10,20].

Despite the potential advantages of dual routes for Asn-tRNA^{Asn} formation, most bacteria encoding AsnRS and GatCAB are predicted to code for a D-AspRS and use GatCAB for just Gln-tRNA^{Gln} formation [6,12,13]. In particular, bacteria are thought to encode a D-AspRS when coding for an Asn synthetase (AsnA and/or AsnB) to synthesize Asn in a tRNA-independent manner and AsnRS to ligate Asn to tRNA^{Asn} [6,12,13]. *Bacillus subtilis* and *Bacillus halodurans* are two such bacteria (Table 1). Both *Bacilli* code for tRNA-independent Asn biosynthesis, AsnRS, one AspRS, and GatCAB but not GlnRS. Accordingly, in *B. subtilis* and *B. halodurans*, AspRS was predicted to be discriminating with the role of GatCAB limited to Gln-tRNA^{Gln} synthesis despite encoding tRNA^{Asn} isoacceptors with U1-A72 base pairs [6,13].

Given the potential advantages of encoding both routes for Asn-tRNA^{Asn} formation [10,18,20], we tested the predicted discriminating nature of the *B.*

Table 1. Number of genes coding for enzymes involved in Asn, Asp-tRNA^{Asp}, Asn-tRNA^{Asn}, and Gln-tRNA^{Gln} formation found in the genomes of bacteria discussed

Species	AsnA	AsnB	GatCAB	GlnRS	AsnRS	AspRS	U1-A72[a]	Predicted[b]	Actual[c]
B. halodurans	**0**	**1**	**1**	**0**	**1**	**1**	**Y**	**D** [13]	**ND**
B. subtilis	**0**	**3**	**1**	**0**	**1**	**1**	**Y**	**D** [6,13]	**ND**
B. bacteriovorus	0	0	1	1	1	1	Y	PD [20]	ND
B. burgdorferi	**0**	**0**	**1**	**0**	**1**	**1**	**Y**	**ND** [20]	**ND**
C. acetobutylicum	0	1 NF[d]	2	0	1	3	Y	PD [18]	2ND, 1D
D. radiodurans	0	0	1	1	1	2	Y	PD [17]	1ND, 1D
E. coli	1	1	0	1	1	1	Y	PD [22]	D
Lactobacillus delbrueckii	1	2	1	0	2	1	Y	PD [9]	D
L. pneumophila	0	0	1	1	1	1	Y	PD [20]	ND
M. pneumoniae	**0**	**0**	**1**	**0**	**1**	**1**	**N**	**D** [13]	**D**
S. aureus	0	0	1	0	1	1	Y	PD [10]	ND
T. thermophilus	0	0	1	1	1	2	Y	PD [16]	1ND, 1D

D for a D-AspRS, ND for an ND-AspRS, and PD for an AspRS with previously determined tRNA specificity. Predictions were based on previous studies of genomic content. The predictions tested and novel results presented are in boldface.

[a] U1-A72 refers to the presence of a U1-A72 base pair in tRNA^{Asn} isoacceptors in the organism.

[b] Predicted discriminating nature of the encoded AspRSs.

[c] Actual discriminating nature of the encoded AspRSs.

[d] NF, non-functional under conditions assayed. The *C. acetobutylicum asnB* homolog is split, and under the conditions grown, no AsnB activity could be detected in the organism [18].

subtilis and *B. halodurans* AspRS enzymes. We found that, in contrast to the predictions based on genomic content, both *Bacilli* AspRSs are non-discriminating and enable tRNA-dependent Asn biosynthesis with GatCAB. Both bacteria therefore encode the direct and indirect routes for Asn-tRNA^{Asn} formation along with tRNA-independent Asn biosynthesis. Our phylogenetic analysis reveals that this may be more common than predicted especially among Firmicutes. In addition, the phylogenetic study suggests that the evolutionary default of bacterial AspRS is non-discriminating with tRNA^{Asp} specificity evolving independently in select bacterial lineages with a similar pattern observed among archaeal AspRS enzymes.

Results

tRNA specificity of the *Mycoplasma pneumoniae*, *Borrelia burgdorferi*, and *B. halodurans* AspRSs

Previous work established that bacteria encoding AsnRS but lacking a functional Asn synthetase could encode an ND-AspRS with GatCAB for tRNA-dependent Asn biosynthesis in species coding for tRNA^{Asn} isoacceptors with a U1-A72 base pair [10,20]. We decided to test whether additional bacteria encode both routes for Asn-tRNA^{Asn} formation. The three species we investigated with were *M. pneumoniae*, *B. burgdorferi*, and *B. halodurans*, which all encode AsnRS, GatCAB, and one AspRS but not GlnRS (Table 1). The first also does no code for either Asn synthetase (Table 1). Like *S. aureus* and *B. bacteriovorus*, *B. burgdorferi* encodes tRNA^{Asn} isoacceptors with a U1-A72 base pair and was predicted to encode an ND-AspRS [20]. In contrast, *M. pneumoniae* encodes tRNA^{Asn} isoacceptors with a G1-C72 pair and is predicted to encode a D-AspRS [13]. *B. halodurans*, while encoding tRNA^{Asn} isoacceptors with U1-A72 base pairs, does also code for an AsnB homolog, AsnO, and was therefore predicted to encode a D-AspRS using its GatCAB solely for Gln-tRNA^{Gln} formation [6,13].

To test those predictions, we used the established *E. coli trpA*34 missense suppressor system [10,20–22]. The strain is a Trp auxotroph due to mutation of codon 60 in the gene for the Trp synthase alpha subunit (*trpA*) from an Asp codon to an Asn codon [23]. The phenotype can be rescued by producing an ND-AspRS in the strain by enabling formation of the Asp-tRNA^{Asn} missense suppressor in *E. coli* [22]. Consistent with our predictions, expression of the *B. burgdorferi aspS* rescued the strain while expression of the *M. pneumoniae aspS* did not (Fig. 1a). As *E. coli* tRNA^{Asn} isoacceptors have a U1-A72 base pair, we also tested *in vitro* whether the *M. pneumoniae* AspRS could recognize its own tRNA^{Asn} (Fig. 1b). As expected, the *M. pneumoniae* enzyme strongly preferred tRNA^{Asp} as a substrate (Fig. 1b) while the *B. burgdorferi* AspRS aminoacylated its tRNA^{Asn} almost as well as tRNA^{Asp} (Fig. 1c).

However, expression of the *B. halodurans aspS* rescued the Trp auxotrophy of the strain (Fig. 2a), indicative of the enzyme being non-discriminating in contrast to its predicted tRNA specificity based on genomic content [13]. To further validate *B. halodurans* AspRS as a non-discriminating aaRS, we assayed *in vitro* how well it could aspartylate tRNA^{Asn} compared to tRNA^{Asp} (Fig. 2b and Table 2). The tRNA^{Asp} was a better substrate than tRNA^{Asn} for the *B. halodurans* AspRS by 5-fold (Table 2). The AspRS had a 2.1-fold lower K_M for tRNA^{Asn} than tRNA^{Asp} but also had a 9.6-fold lower k_{cat}. However, the preference for

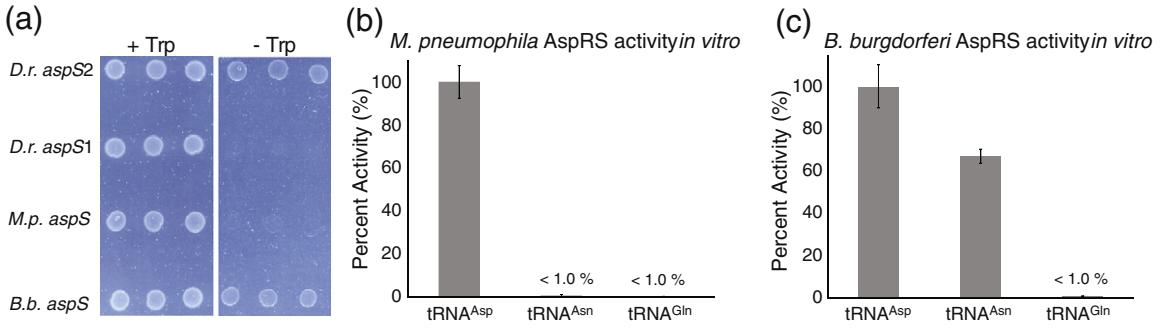

Fig. 1. Discriminating nature of the *M. pneumoniae* and *B. burgdorferi* AspRS enzymes. (a) *E. coli trpA34* was grown with pCBS2 containing (top to bottom) the *ND-aspS* from *D. radiodurans* (*D.r. aspS2*) as a positive control, the *discriminating* (*D*)*-aspS* from *D. radiodurans* (*D.r. aspS1*) as a negative control, the *M. pneumoniae aspS* (*M.p. aspS*), or the *B. burgdorferi aspS* (*B.b. aspS*). The cultures were grown in triplicate on M9 minimal media agar plates with 100 μg/ml of ampicillin in the presence (+Trp, 20 μg/ml) or in the absence (−Trp) of Trp at 37 °C. Aspartylation of *in vitro* transcribed tRNA^{Asp}, tRNA^{Asn}, and tRNA^{Gln} by the (b) *M. pneumoniae* or (c) *B. burgdorferi* AspRS. Reactions were carried out at 37 °C with 0.1 μM ^{32}P-labeled tRNA^{Asp}, tRNA^{Asn}, or tRNA^{Gln} and with 4.0 mM ATP, 4.0 mM L-Asp, and 10 nM AspRS. Experiments were repeated three times and error bars represent standard deviations.

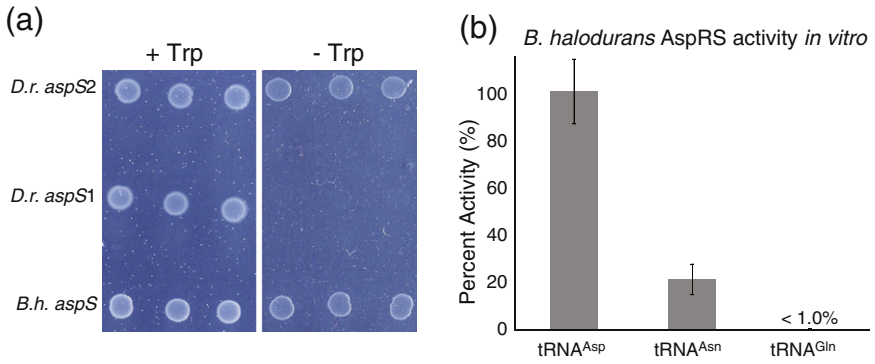

Fig. 2. *B. halodurans* AspRS aspartylates tRNA^{Asn}. (a) *E. coli trpA34* was grown with pCBS2 containing (top to bottom) the *ND-aspS* from *D. radiodurans* (*D.r. aspS2*) as a positive control, the *D-aspS* from *D. radiodurans* (*D.r. aspS1*) as a negative control, or the *B. halodurans aspS* (*B.h. aspS*). The cultures were grown in triplicate on M9 minimal media agar plates with 100 μg/ml of ampicillin in the presence (+Trp, 20 μg/ml) or in the absence (−Trp) of Trp at 37 °C. (b) Aspartylation of *in vitro* transcribed tRNA^{Asp}, tRNA^{Asn}, and tRNA^{Gln} by the *B. halodurans* AspRS. Reactions were carried out at 37 °C with 0.1 μM ^{32}P-labeled tRNA^{Asp}, tRNA^{Asn}, or tRNA^{Gln} and with 4.0 mM ATP, 4.0 mM L-Asp, and 10 nM AspRS. Experiments were repeated three times and error bars represent standard deviations.

tRNA^{Asp} is not unusual. All ND-AspRSs assayed to date display a preference for tRNA^{Asp} over tRNA^{Asn} [10,17,20,24,25]. Similarly, the *Thermotoga maritima* ND-GluRS prefers tRNA^{Glu} to tRNA^{Gln} by 14-fold [26]. In contrast, tRNA^{Asp} is the preferred substrate for D-AspRSs by 500- to 2250-fold [17,24]. The tRNA^{Asn} was a better substrate for the *B. halodurans* AsnRS than it was for AspRS by 5-fold (Fig. 3 and Table 2), similar to other organisms encoding both an ND-AspRS and an AsnRS [10,17,20,24]. For the *B. halodurans* AsnRS, this was due to a 10-fold increase in k_{cat} as compared to AspRS, though the AspRS did have a 1.9-fold lower K_M for tRNA^{Asn} than AsnRS (Table 2).

B. halodurans genes rescue *E. coli* JF448 Asn auxotrophy

Encoding an ND-AspRS along with GatCAB provides *B. halodurans* with a mechanism to synthesize Asn in a tRNA-dependent manner. The organism also encodes AsnO to transamidate Asp to Asn without tRNA. In *C. acetobutylicum*, which also synthesizes Asn on tRNA^{Asn}, its Asn synthetase is split and non-functional under the conditions assayed [18]. To determine if *B. halodurans* encodes two distinct routes to synthesize Asn, we expressed the relevant genes in *E. coli* JF448, an Asn auxotrophic strain due to mutations of its *asnA* and *asnB* genes [17,27]. Expression of the *B. halodurans asnO* rescued the Asn auxotrophy, as did the co-expression of the *B. halodurans aspS* and

Table 2. Aminoacylation kinetics of *B. halodurans* AspRS and AsnRS at 37 °C

	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (s^{-1} μM^{-1})	L[a]
AspRS				
tRNA^{Asp}	0.115 ± 0.004	1.5 ± 0.2	(8 ± 1) × 10^{-2}	5
tRNA^{Asn}	0.012 ± 0.001	0.7 ± 0.3	(1.7 ± 0.6) × 10^{-2}	1
AsnRS				
tRNA^{Asn}	0.12 ± 0.01	1.3 ± 0.3	(9 ± 2) × 10^{-2}	5

[a] L is specificity relative to the catalytic efficiency of AspRS with tRNA^{Asn} as a substrate: (k_{cat}/K_M)/(k_{cat}/K_M) of AspRS for tRNA^{Asn}. Experiments were repeated three to four times and standard deviations are reported.

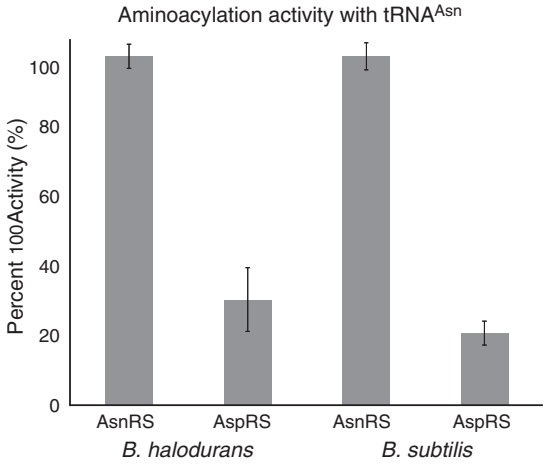

Fig. 3. Aminoacylation of tRNA^{Asn} by *B. halodurans* and *B. subtilis* aaRSs. Aminoacylation of tRNA^{Asn} by AsnRS and AspRS from *B. halodurans* (right) and *B. subtilis* (left). Reactions were carried out at 37 °C with 0.1 μM ^{32}P-labeled tRNA^{Asn}, 4.0 mM ATP, 4.0 mM amino acid, and 10 nM AspRS. The amino acid L-Asn was used for the AsnRS reactions and L-Asp was used in the AspRS reactions. Activities are relative to the homologous AsnRS.

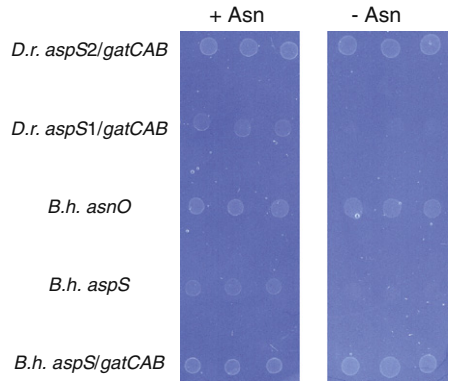

Fig. 4. Production of either *B. halodurans asnO* or *aspS/gatCAB* in *E. coli* JF448 results in an Asn prototroph. *E. coli* JF448 was grown with pCBS2 containing (top to bottom) the *D. radiodurans ND-aspS* and *gatCAB* (*D.r. aspS2*/*gatCAB*) as a positive control, the *D. radiodurans D-aspS* and *gatCAB* (*D.r. aspS1*/*gatCAB*) as a negative control, the *B. halodurans asnO* (*B.h. asnO*), the *B. halodurans aspS* (*B.h. asnS*) alone to control for possible toxic effects of *B. halodurans aspS* expression, or the *B. halodurans aspS* and *gatCAB* in an operon together (*B.h. aspS/gatCAB*). The resultant strains were grown in triplicate on M9 minimal media agar plates with 100 μg/ml of ampicillin in the presence (+Asn, 20 μg/ml) or in the absence (–Asn) of Asn at 37 °C.

gatCAB (Fig. 4), consistent with both systems being able to synthesize Asn.

B. subtilis AspRS aspartylates tRNA^{Asn}

Similar to *B. halodurans*, *B. subtilis* was predicted to encode a D-AspRS as it has an AsnRS for Asn-tRNA^{Asn} formation and can synthesize Asn without tRNA (Table 1) with GatCAB used for Gln-tRNA^{Gln} formation [6,12,13]. Unlike *B. halodurans*, *B. subtilis* encodes three AsnB homologs (AsnB, AsnH, and AsnO), each of which is active with the *B. subtilis* AsnB being the main enzyme for Asn biosynthesis in the organism [28]. We noted that a triple knockout of *asnB*, *asnH*, and *asnO* in *B. subtilis* resulted in a strain that was still an Asn prototroph while slow growing [28]. The results suggest that *B. subtilis* encodes another means to synthesize Asn. Given our results above with *B. halodurans*, we hypothesized in contrast to previous predictions that the lone AspRS in *B. subtilis* is non-discriminating enabling tRNA-dependent Asn biosynthesis. Like *B. halodurans*, the *B. subtilis* tRNA^{Asn} isoacceptors have a U1-A72 base pair suggesting that, if aspartylated, the tRNA^{Asn} could serve as a substrate for GatCAB.

To test tRNA^{Asn} recognition by the *B. subtilis* AspRS, we used the *E. coli trpA*34 and *in vitro* assays described above (Fig. 5). Consistent with the *B. subtilis* AspRS being non-discriminating, production of the enzyme in *E. coli trpA*34 resulted in a Trp prototroph indicative of Asp-tRNA^{Asn} formation (Fig. 5a). Like other ND-AspRSs [10,17,20,24,25], the *B. subtilis* AspRS preferred tRNA^{Asp} to tRNA^{Asn} (Fig. 5b and Table 3). The 6-fold preference was due to improved turnover with tRNA^{Asp} over tRNA^{Asn} (Table 3). The tRNA^{Asn} was a 6-fold better substrate for the *B. subtilis* AsnRS than AspRS (Fig. 3).

An ND-AspRS enables *B. subtilis* to form Asp-tRNA^{Asn}. *In vitro*, the *B. subtilis* GatCAB can transamidate Asp-tRNA^{Asn} to Asn-tRNA^{Asn} [6]. To verify co-production of the *B. subtilis* AspRS and GatCAB that leads to Asn synthesis in an *in vivo* context, we used the *E. coli* Asn auxotrophic strain JF448 complementation assay. Consistent with our hypothesis, co-expression of the *B. subtilis aspS* and

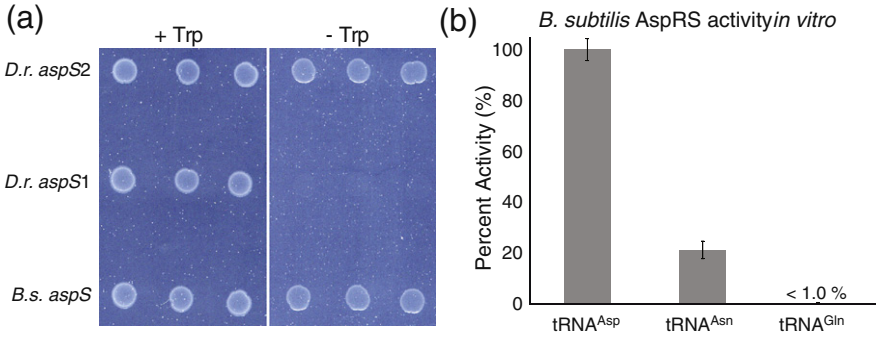

Fig. 5. *B. subtilis* AspRS aspartylates tRNA^{Asn}. (a) *E. coli trpA34* was grown with pCBS2 containing (top to bottom) the *ND-aspS* from *D. radiodurans* (*D.r. aspS2*) as a positive control, the *D-aspS* from *D. radiodurans* (*D.r. aspS1*) as a negative control, or the *B. subtilis aspS* (*B.s. aspS*). The cultures were grown in triplicate on M9 minimal media agar plates with 100 μg/ml of ampicillin in the presence (+Trp, 20 μg/ml) or in the absence (–Trp) of Trp at 37 °C. (b) Aspartylation of *in vitro* transcribed tRNA^{Asp}, tRNA^{Asn}, and tRNA^{Gln} by the *B. subtilis* AspRS. Reactions were carried out at 37 °C with 0.1 μM ^{32}P-labeled tRNA^{Asp}, tRNA^{Asn}, or tRNA^{Gln} and with 4.0 mM ATP, 4.0 mM L-Asp and 10 nM AspRS. Experiments were repeated three times and error bars represent standard deviations.

Table 3. Aminoacylation kinetics of *B. subtilis* AspRS at 37 °C

	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (s^{-1} μM^{-1})	L[a]
AspRS				
$tRNA^{Asp}$	0.36 ± 0.08	1.6 ± 0.6	(22 ± 8) × 10^{-2}	6
$tRNA^{Asn}$	0.058 ± 0.003	1.6 ± 0.3	(4 ± 1) × 10^{-2}	1

[a] *L* is specificity relative to the catalytic efficiency of AspRS with $tRNA^{Asn}$ as a substrate: (k_{cat}/K_M)/(k_{cat}/K_M) of AspRS for $tRNA^{Asn}$. Experiments were repeated three to four times and standard deviations are reported.

gatCAB resulted in an Asn prototroph just similar to expression of the *B. subtilis asnB* in the JF448 strain (Fig. 6).

Evolution of bacterial D-AspRS

The tRNA specificity of an AspRS has typically been predicted based on genomic content [6,12,13,29]. In archaea, the predictions are relatively straightforward as its GatCAB is solely used for Asn-$tRNA^{Asn}$ formation and AsnRS is absent when GatCAB is present [29,30]. In contrast, bacterial GatCAB is used for synthesis of Asn-$tRNA^{Asn}$ and/or Gln-$tRNA^{Gln}$ and can be encoded in the same genome as AsnRS [6,13]. When both AsnRS and GatCAB are encoded in a bacterial genome, the AspRS tRNA specificity is unclear. In bacteria with one AspRS along with GatCAB and AsnRS, the amidotransferase could be used only for Gln-$tRNA^{Gln}$, like is the case in *L. delbrueckii bulgaricus* that has a D-AspRS [9]. However, GatCAB could also be used for both Asn-$tRNA^{Asn}$ and Gln-$tRNA^{Gln}$ formation as is found in *B. halodurans*, *B. subtilis*, and *S. aureus*, which each encode an ND-AspRS in their genomes along with AsnRS.

AsnRS, GatCAB, and one AspRS are encoded in about 32% of bacterial genomes [20]. About 2% of bacteria, such as *M. pneumoniae*, can be predicted to encode a D-AspRS as their $tRNA^{Asn}$ isoacceptors have G1-C72 base pair instead of the U1-A72 required for bacterial tRNA-dependent Asn biosynthesis. However, in most 30% of all bacteria, the tRNA specificity of the AspRS cannot be predicted based on genomic content [20]. Traditionally, these bacteria such as *B. halodurans*, *B. subtilis*, and *S. aureus* are predicted to encode a D-AspRS as they have an AsnRS to aminoacylate $tRNA^{Asn}$ [6,12,13].

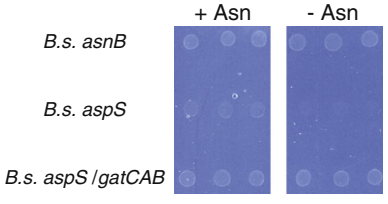

Fig. 6. Co-production of the *B. subtilis aspS* with *gatCAB* in *E. coli* JF448 results in an Asn prototroph. *E. coli* JF448 was grown with pCBS2 containing (top to bottom) the *B. subtilis asnB* (*B.s. asnB*) as a positive control, the *B. subtilis aspS* alone as a negative control, or the *B. subtilis aspS* and *gatCAB* in an operon together (*B.s. aspS/gatCAB*). The resultant strains were grown in triplicate on M9 minimal media agar plates with 100 μg/ml of ampicillin in the presence (+Asn, 20 μg/ml) or in the absence (−Asn) of Asn at 37 °C.

Given that those three actually encode an ND-AspRS, we sought to determine if the predictions could be improved by phylogenetic analysis using a diverse range of bacterial, archaeal, and eukaryotic AspRS and AsnRS sequences. The class II lysyl-tRNA synthetases (LysRS) served as the outgroup to root the phylogeny. The resulting phylogeny displayed a three-domain topology seen previously with AspRS phylogenies (Fig. 7a) [29,31,32]. One previous phylogeny modeled AsnRS evolving from a gene duplication event of the AspRS in a common ancestor of archaea and eukaryotes after the split with bacteria [31]. However, AsnRS appears to have evolved later due to a gene duplication of an early archaeal AspRS and was acquired in different lineages by horizontal gene transfer (Fig. 7a), consistent with other AspRS/AsnRS phylogenies [29,32]. AsnRS evolving after the archaeal/eukaryotic split and the presence of GatCAB in last universal common ancestor (LUCA) [33] strongly suggests that Asn-$tRNA^{Asn}$ formation in LUCA was by the indirect two-step pathway with the ancestral AspRS being non-discriminating. Evolution of D-AspRSs likely arose after AsnRS evolved to aminoacylate $tRNA^{Asn}$.

To determine if D-AspRS evolved once in archaeal-type (Fig. 7b) and bacterial-type (Fig. 7c) AspRSs, we mapped onto the phylogeny the tRNA specificity of AspRSs (D-AspRS or ND-AspRS) based on known biochemical results or clear predictions as to their specificity. A D-AspRS was predicted when AsnRS was present in the organism but GatCAB was not or in bacteria encoding AsnRS and GatCAB but with $tRNA^{Asn}$ isoacceptors lacking the U1-A72 base pair required for bacterial GatCAB recognition. An ND-AspRS was predicted when AsnRS was absent but GatCAB was present in the organism. AspRSs without a clear prediction as to their tRNA specificity were denoted accordingly. D-AspRSs were paraphyletic in both archaeal-type (Fig. 7b) and bacterial-type (Fig. 7c) AspRS clades, suggesting that D-AspRS evolved independently multiple times in both archaea and bacteria.

Among the archaeal AspRSs, there was a split between the Euryarchaea and Crenarchaea (Fig. 7b). A number of Desulfurococcales were

placed outside the Crenarchaea and were grouped with *Nanoarchaeum equitans* and the Euryarchaeon *Halobacterium salinarum*. The AspRS2s found in *C. acetobutylicum* and Deinococcus–Thermus phylum also were part of this clade. The grouping may be due to long-branch attractions. As AsnRS evolved after the split between archaea and eukaryotes, early archaea likely encoded an ND-AspRS, with D-AspRS evolving in lineages that acquired the gene for AsnRS. Within the Euryarchaea clade, the Thermococcaceae AspRS evolved to be specific for $tRNA^{Asp}$ while, in the Crenarchaea, D-AspRS evolved in the Thermoplasmatales.

Early bacteria lacking an AsnRS likely encoded an ND-AspRS and a GatCAB to synthesize Asn on $tRNA^{Asn}$ [33]. It appears that, in most cases, AspRS was vertically inherited within bacterial lineages (Fig. 7c). The notable exceptions were the alpha-proteobacteria and delta-proteobacteria, which separately grouped outside the other proteobacteria possibility indicative of horizontal gene transfer of *aspS* between bacteria. D-AspRS independently evolved in the Mollicutes, the Deinococcus–Thermus phylum, certain lineages of Firmicutes, Bacteroidetes, and the clade of gamma-proteobacteria including Enterobacteriales, Pasteurellales, Vibrionales, and Alteromonadales. As the other gamma-proteobacteria along with the beta-proteobacteria and epsilon-proteobacteria encode ND-AspRSs, likely the common ancestor of gamma-proteobacteria coded for an ND-AspRS. Later acquisition of AsnRS likely enabled the AspRS in the common ancestor of the subset of gamma-proteobacteria to evolve a D-AspRS.

Given the distribution of ND-AspRSs in the bacterial AspRS phylogeny and within the Firmicutes (Fig. 7c), the early common ancestor of the later phylum likely encoded an ND-AspRS. Two lineages of Firmicutes are known to encode D-AspRS enzymes, *C. acetobutylicum* and *L. delbrueckii bulgaricus*. In addition, the former encodes two archaeal ND-AspRSs and GatCABs, as well as AsnRS, while the latter codes for two AsnRSs for Asn-$tRNA^{Asn}$ formation and AsnA along with two AsnB homologs for Asn biosynthesis (Table 1) [9,18]. As the D-AspRS from *C. acetobutylicum* and *L. delbrueckii bulgaricus* are most closely related to ND-AspRSs than they are to one another suggests that each AspRS independently evolved specificity for $tRNA^{Asp}$. In the majority of Firmicutes, the discriminating nature of the AspRS is not clear as the organisms encode both an AsnRS and a GatCAB along with $tRNA^{Asn}$ with a U1-A72 base pair. Given that D-AspRS evolution is lineage specific, the phylogeny cannot clarify the discriminating nature of these AspRS enzymes, similar for other bacterial AspRSs with unknown tRNA specificities. The exception may be the bacterial lineages encoding AsnRS and GatCAB whose AspRSs nested within the D-AspRS clades of the gamma-proteobacteria and Bacteroidetes, which suggests that they may also encode D-AspRSs.

Discussion

ND-AspRS and GatCAB for tRNA-dependent Asn biosynthesis

M. pneumoniae encodes a D-AspRS and uses GatCAB likely just for Gln-$tRNA^{Gln}$ synthesis consistent with its $tRNA^{Asn}$ isoacceptors having G1-C72 base pairs. AsnRS likely is the only aaRS in the species that aminoacylates $tRNA^{Asn}$. Given that *M. pneumoniae* does not encode either Asn synthetase, the species is likely an Asn auxotroph unless it codes for a novel Asn biosynthetic pathway. *B. burgdorferi*, like *S. aureus*, *L. pneumophila*, and *B. bacteriovorus* [10,20], encodes a single AspRS that is non-discriminating along with AsnRS. As these bacteria also encode GatCAB but not AsnA and AsnB, the ND-AspRS with GatCAB allows the species to synthesize Asn on $tRNA^{Asn}$. The AsnRS in these bacteria may enable these species to exploit environments where Asn is plentiful such as when they are growing in a host organism [10,20].

Retention of an ND-AspRS in *B. halodurans* and *B. subtilis*

Contrary to predictions based on genomic content [6,12,13], *B. halodurans* and *B. subtilis* also encode an ND-AspRS along with AsnRS, GatCAB, and the means to synthesize Asn without tRNA. Their ND-AspRSs with GatCABs can be used for tRNA-dependent Asn synthesis. Thus, *B. halodurans* and *B. subtilis* code for tRNA-dependent Asn biosynthesis despite encoding AsnB to synthesize Asn and AsnRS to aminoacylate the amino acid to $tRNA^{Asn}$.

The role of tRNA-dependent Asn biosynthesis in *B. halodurans* and *B. subtilis* is not clear. Recognition of $tRNA^{Asn}$ by the *B. halodurans* and *B. subtilis* AspRS enzymes may be vestigial. As AsnRS appears to have evolved after the emergence of the three domains of life, early bacteria likely used an ND-AspRS and a GatCAB to synthesize Asn-$tRNA^{Asn}$ [33]. Accordingly, bacterial lineages that acquired AsnRS likely initially also had an ND-AspRS to aminoacylate $tRNA^{Asn}$ isoacceptors with U1-A72 base pairs to be recognized by GatCAB [13]. In bacteria, like the ancestor of many gamma-proteobacteria that also acquired GlnRS for Gln-$tRNA^{Gln}$ formation, GatCAB became dispensable and AspRS evolved to be specific for $tRNA^{Asp}$. However, bacteria such as *B. halodurans* and *B.*

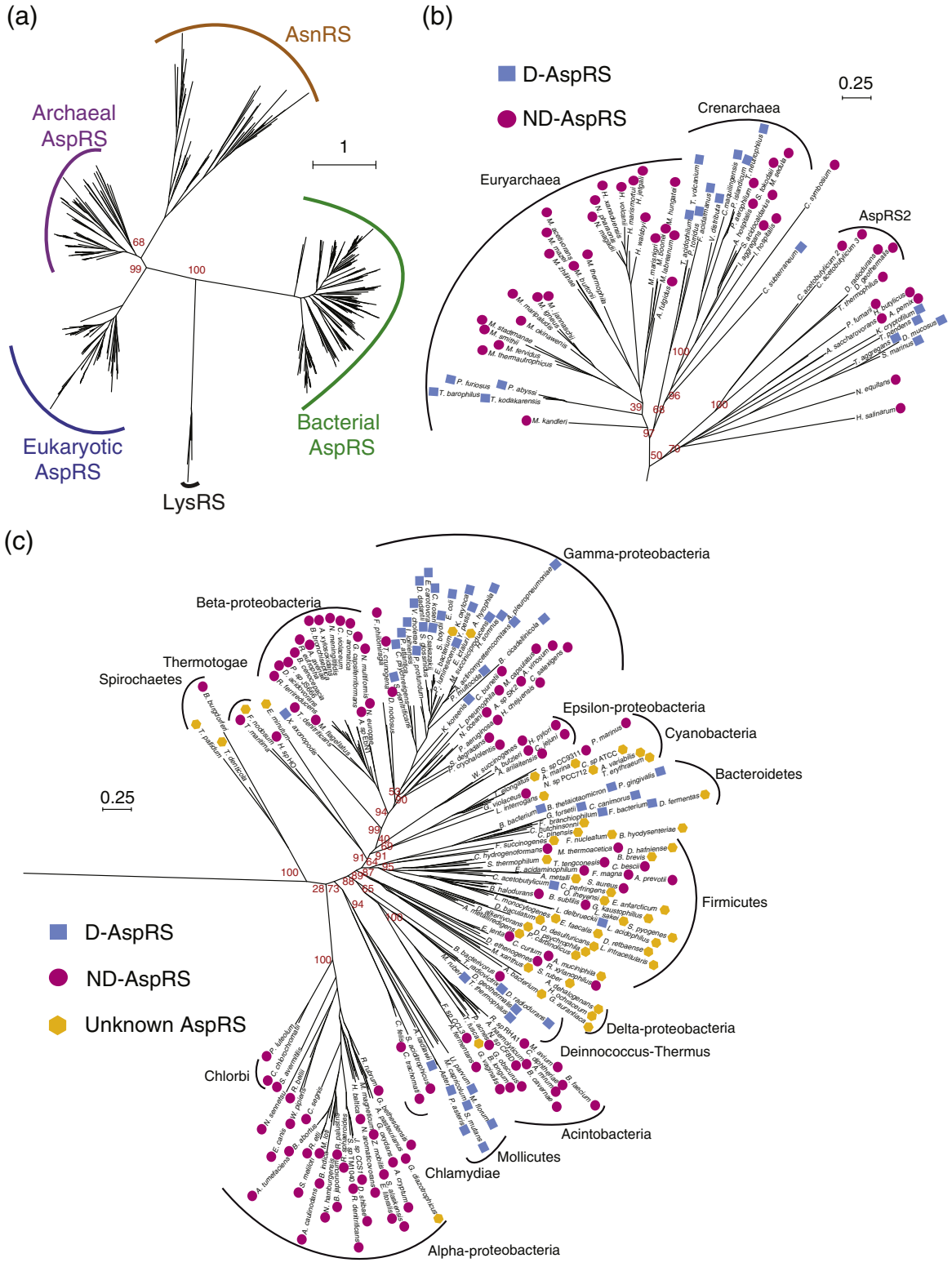

Fig. 7 *(legend on next page)*

subtilis never acquired a GlnRS and retained GatCAB for Gln-tRNA^{Gln} formation. In these species, AspRS may never have been under a selective pressure to evolve tRNA specificity as any Asp-tRNA^{Asn} formed by the ND-AspRS could be transamidated to Asn-tRNA^{Asn} by GatCAB.

However, it does appear in *B. subtilis* enough that Asn is synthesized on tRNA^{Asn} by the ND-AspRS and GatCAB to sustain life. Knocking out the three genes encoding AsnB homologs in *B. subtilis* results in a strain that is slow growing but able to grow in the absence of Asn [28]. As *B. subtilis* does not encode AsnA, the results are consistent with tRNA-dependent Asn synthesis by the ND-AspRS and GatCAB providing a basal level of Asn for the organism. Growth of the triple knockout strain does improve in the presence of Asn (5 mg/ml) slightly more than the wild-type strain (2.1-fold improvement *versus* 1.5-fold, respectively) [28].

Using GatCAB for Asn-tRNA^{Asn} formation in *B. subtilis* would explain the excess production of the amidotransferase relative to ND-GluRS in the organism during exponential and early stationary growth phases (2.4-fold and 2.3-fold, respectively) [34]. Bacterial GatCAB forms complexes known as transamidosomes with either ND-GluRS or ND-AspRS [35–41]. A higher concentration of GatCAB than ND-GluRS would enable the formation of transamidosomes between ND-AspRS and GatCAB for Asn-tRNA^{Asn} formation. Under the same conditions, GatCAB is also in excess of ND-AspRS by 1.5-fold and 2.3-fold, respectively, indicating that transamidosomes between the ND-GluRS and GatCAB for Gln-tRNA^{Gln} formation can also form [34]. During late stationary phase, GluRS production is 1.8-fold greater than GatCAB while the amidotransferase is produced in excess by 1.9-fold compared to the ND-AspRS suggesting that GatCAB under those conditions is used more for Gln-tRNA^{Gln} formation [34]. AsnRS production during late stationary growth is also in excess of AspRS presumably to synthesize Asn-tRNA^{Asn}, while during exponential and early stationary phases, AsnRS production is similar to that of AspRS.

Dual routes for aminoacylating a tRNA

Encoding more than one enzyme to aminoacylate a tRNA is not unusual in *Bacilli*. *B. subtilis* encodes two threonyl-tRNA synthetases (ThrRSs) and two tyrosyl-tRNA synthetases (TyrRSs) [42,43]. The relevant genes are under the control of T-box riboswitches [44]. For ThrRS, the second enzyme (ThrZ) is produced when there is increased demand for Thr-tRNA^{Thr} formation [42]. In the case of TyrRS, the additional enzyme (TyrZ) appears to protect the organism from misincorporation of D-Tyr [43]. It has been suggested the indirect pathway for Asn-tRNA^{Asn} formation under certain conditions may provide a higher degree of fidelity than the direct route with AsnRS [5]. *Bacillus cereus* encodes both class I and class II lysyl-tRNA synthetases (LysRSI and LysRSII) [45]. Together, the two LysRSs can aminoacylate a non-canonical tRNA [45]. LysRSII is produced in both exponential and stationary phases while LysRSI is produced only in the stationary phase. LysRSI is regulated by a T-box riboswitch [46]. In. *B. subtilis* and *B. halodurans*, for Asn-tRNA^{Asn} synthesis, only *aspS* is under the control of a T-box riboswitch [44].

Among Firmicutes, three are known to encode more than one enzyme to aminoacylate tRNA^{Asn}. *L. delbrueckii bulgaricus* encodes two AsnRSs for reasons that are currently unknown [9]. *C. acetobutylicum* has an AsnRS and two ND-AspRSs along with a D-AspRS and two GatCABs [18,19]. The redundancy is thought to be involved in the organism switching between acidogenesis to solventogenesis with regulation by T-box riboswitches [18]. *S. aureus* encodes one ND-AspRS and GatCAB along with AsnRS [10]. The indirect route is thought to provide the sole means for Asn biosynthesis in the organism with the AsnRS present to exploit environments rich in Asn and recycle Asn from protein degradation. In other bacteria encoding an ND-AspRS, GatCAB, and AsnRS but neither Asn synthetase, the direct and indirect routes for Asn-tRNA^{Asn} synthesis are thought to play similar roles [3,10,17,20]. Similarly, archaea encoding both direct and two-step indirect routes for Cys-tRNA^{Cys} formation use the two-step pathway as the sole means for Cys biosynthesis with the direct route recycling Cys from protein degradation [2]. In *Methanosarcina mazei*, the dual routes have been further implicated in cellular physiology and each route with a distinct set a tRNA^{Cys} isoacceptors [47].

In *B. subtilis* and *B. halodurans*, the two routes for Asn-tRNA^{Asn} formation may also enable adaptation to different environments though their tRNA^{Asn} isoacceptors are highly similar. Given that a number of other Firmicutes encode AsnRS and GatCAB with tRNA^{Asn} isoacceptors with a U1-A72 base pair, it

Fig. 7. Phylogeny of the Asp/AsnRS protein family. (a) Phylogeny of AspRS and AsnRS sequences from eukaryotes, archaea, and bacteria with class II LysRS sequences serving as the outgroup. Scale bar represents 1 change per site. (b) Enlargement of the phylogeny in (a), focusing on the archaeal AspRS sequences. To better differentiate the lineages, we rotated branches relative to (a). Scale bar represents 0.25 changes per site. (c) Enlargement of the phylogeny in (a), focusing on the bacterial AspRS sequences. To better differentiate the lineages, we rotated branches relative to (a). For (a), (b), and (c), only bootstrap values for the branch points discussed in the text are shown for clarity. For (b) and (c), violet circles are for D-AspRSs, blue squares are for ND-AspRSs, and gold octagons are for AspRSs with unknown tRNA specificities.

may be common in the phylum for their AspRSs to be non-discriminating and thus to encode both routes for Asn-tRNA^{Asn} formation in addition to using GatCAB for Gln-tRNA^{Gln} synthesis. The known exceptions are *L. delbrueckii bulgaricus* and *C. acetobutylicum* [9,18]. However, in the former case, it encodes two AsnRSs, unusual within the clade [9]. *C. acetobutylicum* is also unusual in that it encodes two archaeal-type ND-AspRSs [18] in addition to a bacterial-type D-AspRS.

Convergent evolution of D-AspRS

As noted above, early bacteria likely used an ND-AspRS with GatCAB to synthesize Asn on tRNA^{Asn} with AsnRS acquired from archaea later in certain bacterial lineages [33]. This is consistent with the retention of the important bacterial GatCAB recognition element, the U1-A72 base pair, in tRNA^{Asn} isoacceptors in 87% of bacteria that do not even encode the amidotransferase and directly ligate Asn to tRNA^{Asn} using AsnRS [20]. Evolution of a G1-C72, as is found in *M. pneumoniae* and other Mollicutes, is uncommon and may be due to chance rather than selective pressure.

For an AspRS to evolve specificity for tRNA^{Asp}, AsnRS must be present in the organism to ensure that tRNA^{Asn} is aminoacylated and Asn can be used in protein synthesis. As AsnRS acquisition was lineage specific, evolution of a bacterial-type D-AspRS from an ND-AspRS happened multiple times independently in bacteria, similarly in archaea as well. Acquisition of an AsnRS also did not mean that an organism evolved to have a D-AspRS as found in *B. bacteriovorus* [20], *S. aureus* [10], *B. burgdorferi*, *B. halodurans*, and *B. subtilis*. Organisms that lost GatCAB due to acquiring GlnRS and AsnRS such as a number of gamma-proteobacteria, evolving a D-AspRS, may have been selected to avoid the build-up of Asp-tRNA^{Asn}, which can be toxic [25]. In bacteria such as the Mollicutes that acquired an AsnRS but retain GatCAB for Gln-tRNA^{Gln} synthesis, evolution of AspRS specificity for tRNA^{Asp} may have been selected to enable GatCAB to be used for only Gln-tRNA^{Gln} synthesis.

Retention of the link between intermediary metabolism and translation

Bacteria such as *B. halodurans* and *B. subtilis* that have retained an ND-AspRS and thus tRNA-dependent Asn biosynthesis have kept a direct link between synthesizing Asn and using the amino acid in protein synthesis. The link likely dates back to before the split in LUCA [33] and may have been the mechanism by which Asn was added to the genetic code. Comparing the coupling of Asn and Asn-tRNA^{Asn} biosynthesis to separately synthesizing and ligating the amino acid to tRNA^{Asn} will likely provide insight into how bacteria have adapted to distinct niches in the environment and may improve metabolic modeling of microbial communities [48]. Such insight would also explain why many species retained the indirect route for Asn-tRNA^{Asn} synthesis while others replaced it with the direct one using AsnRS.

Materials and Methods

General

Oligonucleotides were obtained from Integrated DNA Technologies (San Diego, CA) and samples were sequenced at the Yale DNA Analysis Facility on Science Hill (New Haven, CT). *B. halodurans*, *M. pneumoniae*, and *B. burgdorferi* genomic DNA and *B. subtilis subtilis* 168 were from ATCC (Manassas, VA). Nuclease P1 and amino acids were from Sigma-Aldrich (St. Louis, MO). Phenol, NTPs, and chloroform were from Fisher Scientific (Pittsburg, PA). [α-^{32}P]ATP (10 mmol/μCi) was from Perkin Elmer (Shelton, CT). Polyethyleneimine-cellulose thin-layer chromatography glass plates were from EMD Millipore (Billerica, MA). Relevant restriction enzymes, *E. coli* NiCo21(DE3) and NEB10β strains, OneTaq DNA Polymerase, and T4 DNA ligase were from New England Biolabs (Ipswich, MA). *E. coli* JF448 was from the Yale Coli Genetic Stock Center (New Haven, CT), while the *E. coli trpA*34 was a gift from the Söll Laboratory at Yale University (New Haven, CT).

E. coli trpA34 in vivo assay

The *B. halodurans*, *B. subtilis*, *M. pneumoniae*, and *B. burgdorferi aspS* genes were separately subcloned into pCBS2 between the NdeI and BglII restriction sites [22]. Following transformation into *E. coli trpA*34 cells, we grew the cultures and assayed them as described previously on M9 minimal media agar plates with or without Trp with minor adjustments [10]. Briefly, cultures were grown overnight at 37 °C in LB in the presence of ampicillin (100 μg/ml). Cell cultures (1.0 ml) were spun down at 4000 rpm for 10 min to pellet the cells. The cell pellets were then resuspended in 0.5 ml of M9 minimal media before being spun at 4000 rpm for 10 min to re-pellet the cells. The cell pellets were once again resuspended in 0.5 ml of M9 minimal media before spotting 2 μl of culture on M9 minimal media agar ampicillin (100 μg/ml) plates with or without L-Trp (20 μg/ml) and were supplemented with the other 19 amino acids (20 μg/ml each). The plates were then grown overnight at 37 °C.

E. coli JF448 *in vivo* assay

The *B. halodurans aspS* and *gatCAB* were fused into an artificial operon as described previously [22] and subcloned into the pCBS2 plasmid between the NdeI and BglII restriction sites (pCBS2-*Bb-aspS-gatCAB*) and transformed into *E. coli* JF448 cells. Similarly, the *B. subtilis aspS* and *gatCAB* were subcloned together as an artificial operon into pCBS2 and then transformed into *E. coli* JF448 cells. The *B. halodurans asnO* and *B. subtilis asnB* were

also separately subcloned into pCBS2 and transformed into *E. coli* JF448. The cells were grown and assayed as described previously on M9 minimal media agar plates with or without Asn with some minor adjustments [10]. Briefly, cultures were grown overnight at 37 °C in LB in the presence of ampicillin (100 μg/ml). Cell cultures (1.0 ml) were spun down at 4000 rpm for 10 min to pellet the cells. The cell pellets were then resuspended in M9 minimal media (1.0 ml) before being spun at 4000 rpm for 10 min to re-pellet the cells. The cell pellets were once again resuspended in M9 minimal media (1.0 ml). Samples then diluted to an OD_{600} of 0.45 with M9 minimal media. The samples were then diluted 100-fold in M9 minimal media before spotting culture (2.0 μl) on M9 minimal media agar ampicillin (100 μg/ml) plates with or without L-Asn (20 μg/ml) and were supplemented with the other 19 amino acids (20 μg/ml each). The plates were grown at 37 °C overnight.

Over-production and purification of aaRSs

The *B. halodurans*, *B. subtilis*, *M. pneumoniae*, and *B. burgdorferi aspS* genes were chemically synthesized with optimized codons for protein production in *E. coli* (Life Technologies, GeneArt) and then subcloned between the NdeI and BamHI sites in pET28a to be N-terminally His_6 tagged. The proteins were over-produced and purified by nickel-affinity chromatography as described for the *S. aureus* AspRS [10]. For the *B. halodurans* AspRS purification, the Hepes–KOH buffer was adjusted to pH 8.0 throughout the purification process.

The *B. halodurans* and *B. subtilis* genes encoding AsnRS were chemically synthesized and subcloned between the NdeI and BamHI sites in pET28a to be N-terminally His_6 tagged. The proteins were over-produced and purified by nickel-affinity chromatography as described for the *S. aureus* and *B. bacteriovorus* AsnRS enzymes.

In vitro transcription, tRNA folding, and ^{32}P labeling

The relevant tRNA genes were chemically synthesized from Integrated DNA Technologies with a T7 promoter and BstNI restriction site on the 3′ end. For the $tRNA^{Asn}$ isoacceptors with a U1-A72 base pair, a hammerhead ribozyme was coded for between the T7 promoter and the tRNA gene as described previously [30,49]. The plasmids encoding the tRNAs were digested with BstNI followed by phenol/chloroform extraction and ethanol precipitation. The digested DNA samples were resuspended in autoclaved dH_2O before serving as the templates for *in vitro* transcription as previously described. The tRNA samples were purified by gel electrophoresis as previously described. The tRNAs were heated to 95 °C for 5 min and slowly cooled to 65 °C when $MgCl_2$ was added to a final concentration of 5 mM. The tRNA samples were then slowly cooled to room temperature to refold. Samples were stored at −20 °C and ^{32}P-labeled as described previously using the *E. coli* CCA-adding enzyme [12].

^{32}P-based tRNA aminoacylation assay

The aminoacylation activities of the aaRSs were monitored using the established ^{32}P-based assay [12,40,50–52]. The *B. subtilis*, *M. pneumoniae*, and *B. burgdorferi* AspRS reactions contained 50 mM Hepes–KOH (pH 7.2), 30 mM KCl, 15 mM $MgCl_2$, 5 mM DTT, 4 mM L-Asp, and 4 mM ATP. The *B. halodurans* conditions were the same except that the pH of the buffer was 8.0. For enzyme activity, assays with 10 nM AspRS were carried out at 37 °C with 100 nM ^{32}P-labeled tRNA. For the steady-state kinetic studies, assays with 10 nM AspRS were carried out at 37 °C with 0.055–1.0 μM ^{32}P-labeled tRNA and 0–10.0 μM tRNA over 6 min. Reaction mixtures and enzymes were pre-incubated for 30 s at 37 °C and were started by adding enzyme to the reaction mixture. Time points were quenched, digested, separated by thin-layer chromatography, processed, and analyzed in KaleidaGraph 4.5 as described previously [10,12,40,52]. The conditions for the reactions with the *B. subtilis* and *B. halodurans* AsnRS enzymes were the same as they were for the homologous AspRS enzyme except that L-Asp was replaced with L-Asn (4 mM).

Bioinformatic survey of bacterial genomes

AspRS, AsnRS, and LysRS sequences were obtained from either in the UniProt[‡] or KEGG: Kyoto Encyclopedia of Genes and Genomes[§] databases. Sequences were then aligned and manually refined in Geneious 4.7.4. The alignment was then used for phylogenetic analysis as previously described [53] using PhyML [54].

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†N.N. and H.R. contributed equally to this work.
‡http://www.uniprot.org.

§http://www.genome.jp/kegg/.

Abbreviation used:
LUCA, last universal common ancestor.

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